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14. ABSTRACT
Inhibitors of growth factor receptors such as trastuzumab (herceptin) or lapatinib inhibit downstream signaling kinases including mTOR which it is predicted would induce the cells to undergo autophagy. While autophagy may eventually result in cell death, for many days it can recycle intracellular molecules, generating ATP to support cell viability. Hence, it is hypothesized that autophagy will protect tumor cells from trastuzumab and lapatinib. Inhibitors of autophagy would then represent an effective adjuvant to enhance cell killing. It was observed that lapatinib induced autophagy in BT474 breast cancer cells. Autophagy was effectively suppressed using shRNA targeted to ATG12. Lapatinib was shown to completely suppress cell growth, but the cells appeared to survive in a quiescent state with no evidence of cell death. Cells suppressed for autophagy showed no evidence of additional cell death, although evidence was obtained that ATG12 protein levels may have recovered, thereby compromising this experiment. The results obtained are consistent with the original hypothesis that inhibitors of growth factor receptors will induce autophagy. However, we have been unable to confirm that autophagy protects the cells from these drugs which does not support the original hypothesis.

15. SUBJECT TERMS
Breast cancer, trastuzumab, lapatinib, autophagy, cancer chemotherapy

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INTRODUCTION:
Trastuzumab (Herceptin) is considered an effective therapy for breast cancers that over-express the Her2/neu receptor. However, many patients do not respond even when the appropriate target is over-expressed. This research proposes an explanation for these failed therapies and a strategy to improve therapeutic outcome. Inhibition of growth factor receptors inhibits signaling through Akt and mTOR which may induce apoptotic cell death of tumor cells, but inhibition of mTOR also activates autophagy, an alternate survival pathway in which a cell "eats" itself to provide the necessary energy to survive. Accordingly, cells incubated with receptor inhibitors can survive for several weeks, only to recover between periods of drug administration. Therapy would be greatly enhanced if this drug-induced stasis could be converted into cell killing. Accordingly, we hypothesize that autophagy limits the therapeutic efficacy of trastuzumab and other inhibitors of receptor-mediated signal transduction. If this hypothesis is confirmed, then it would justify the development and testing of inhibitors of autophagy to enhance the cancer cell killing by these agents. The original objectives of this project were to generate breast cancer cell lines that are suppressed for autophagy using shRNA technology, and to compare trastuzumab-mediated cell killing in the presence or absence of autophagy using cell culture models and breast cancer tumor xenografts.

BODY

Task 1: To generate cell lines suppressed for autophagy

Three different lentivirus constructs expressing shRNA targeting the autophagy gene ATG5 were purchased from Open Biosystsems. These lentiviral constructs were packaged by passage through HEK293 cells. BT474 cells were infected, selected for expression of the marker gene (G418 resistance), and then assayed for expression of ATG5. The resulting cells show strong suppression of the target protein (Fig. 1 bottom panel). As discussed further under Task 3, trastuzumab did not induce accumulation of autophagosomes as judged by increased expression of LC3-II (this is the lipidated form of LC3 that forms in the autophagosome membrane and which is used as a common marker of autophagy). The accumulation of LC3-II can be detected by incubation of the cells with bafilomycin (Fig. 1, top panel), and this occurred independent of incubation with trastuzumab. Importantly, in the cells suppressed for ATG5, bafilomycin still induced accumulation of LC3-II demonstrating that the suppression of ATG5 was insufficient to prevent autophagy (Fig. 1 bottom).

As a second approach to inhibit autophagy, we purchased several shRNA vectors targeting ATG12 from Open Biosystems and generated BT474 cells suppressed for ATG12. Successful suppression of the target protein was obtained. We studied two clones in more detail (Fig. 2). ATG12 and ATG5 form a covalent heterodimer in cells. In one of the clones (#2), partial suppression of ATG12 resulted in the appearance of monomeric ATG5 as further evidence that ATG12 was now limiting. Surprisingly, the other clone (#1) showed much greater suppression of ATG12 but no accumulation of ATG5 suggesting that the suppression had deleted both proteins. This could occur if ATG12 is required to stabilize ATG5 and any residual monmeric ATG5 would be degraded. Hence, the observations of these two clones is somewhat disparate, although it was clear that ATG12 expression was suppressed. Incubation of parent cells with bafilomycin caused a dramatic accumulation of LC3-II as indication of autophagy, and this was almost completely suppressed in shRNA-expressing clone #1 which had the greatest suppression of ATG12 (Fig. 2). However, as discussed below, we found that ATG12 recovered over time which complicated our ability to resolve the importance of autophagy in response to growth factor receptor-targeted inhibitors.

In experiments discussed below, we also found that BT474 cells grow very slowly and this has made it difficult to discriminate the impact of suppression of autophagy. We have recently obtained more promising results with SKBR3 cells and are currently infecting these cells with the shRNA constructs with the intent of repeating these experiments in another cell line.

Task 2: To generate cells lines engineered to express luminescent markers of autophagy

Soon after submitting this proposal, a paper was published showing that LC3-II forms oligomers in the autophagosome membrane. This fact modified our approach as we could now anticipate obtaining a much more selective signal for autophagy using bioluminescent resonant energy transfer (BRET). Accordingly, we generated two fusion proteins one of which fused luciferase to LC3-II, while the other fused a green fluorescent protein (GFP) to LC3-II. When LC3-II oligomerizes, the two proteins will be in very close proximity such that the light generated by luciferase will induce GFP fluorescence, and hence a very selective fluorescent signal. However while generating these fusion proteins, our concurrent experiments identified a limitation with this approach. We originally expected that LC3-II would accumulate in cells undergoing autophagy. However, it then became apparent that autophagy is a very active process, and that autophagy is characterized by rapid flux through the pathway rather than significant accumulation of autophagosomes. This explains why many publications actually see very little LC3-II accumulating at any given time point. To be able to see the accumulation, it is usually necessary to inhibit the process with agents such as bafilomycin that prevent the turnover of the autophagosomes (See Figs. 1 and 2). This approach would not be possible *in vivo* which was the goal of these experiments. We therefore postponed further work on this task to focus on analysis of autophagy in vitro as in Task 3.

Task 3: To determine the impact of autophagy in the response of cells to trastuzumab *in vitro*

The majority of research on this project addressed this task. The overall hypothesis of this research was that the Her2/neu inhibitor trastuzumab would induce autophagy. This was predicated on the knowledge that trastuzumab inhibits signaling through the AKT/mTOR pathway, and that inhibition of mTOR would induce autophagy. However, experiments in Fig. 1 failed to show the anticipated induction of autophagy. We therefore performed numerous experiments to investigate the anticipated suppression of phospho-AKT as evidence this pathway is inhibited. Experiments covered a large range of concentrations and time points up to 96 h. We found only very transient inhibition in contradiction to what has been published in a number of papers. For example, incubation with 6, 2 or 0.6 μg/ml trastuzumab for 0-48 h showed only transient and weak suppression of phosphorylation of AKT on both serine 473 and threonine 308 (Fig. 3). We questioned whether these results might be attributable to the stimulatory affect of serum in media, but cells incubated at 1% or 0.1% serum showed almost identical results to those in 10% serum (compare Fig. 4 to Fig 3). Using the ZR75.1 cell line which also express Her2/neu, we also failed to see suppression of phospho-AKT (Fig. 5). Interestingly, when we presented these results at the DOD-sponsored conference in Baltimore, several of the other presenters confirmed that they had similar observations and that suppression of Her2/neu frequently does not significantly inhibit the pathway in cell culture. Consistent with our observations, the failure to inhibit phospho-AKT completely or partially for more than 16 h correlated with the lack of any detectable autophagy (Fig. 1). Regretably, a considerable amount of time and effort was expended before we determined that this was not a viable approach for this research project.

To overcome the problems with trastuzumab, we obtained lapatinib from GlaxoSmithKline. Lapatinib has the advantage that it inhibits both Her2/neu and EGFR, and as a consequence we found it induced a complete and prolonged inhibition of phospho-AKT. We made one very important observation in our initial experiments. When BT474 cells (and subsequently confirmed in SKBR3 cells) were incubated with 20 nM lapatinib, we observed prolonged suppression of phospho-EGFR and phospho-Her2 consistent with inhibition of the targeted receptors (Fig. 6). Concurrently we observed suppression of phospho-AKT and phospho-ERK, but in the latter case, this was very transient and by 8 h, phospho-ERK had increased again; by 24 h, the level of phospho-ERK far exceeded even the starting level. This observation may have a significant impact on the efficacy of lapatinib in patients. Incubation with 1 μM lapatinib was far more effective at suppressing both phospho-ERK and phospho-AKT over the full 72 h period.

Cells incubated with either 20 nM or 1 μM lapatinib showed an accumulation of LC3-II within 2 h of administration of the drug, with some increase thereafter although the level appeared to plateau by 6-8 h (Figs. 7 and 8). This fold increase is less than observed upon incubation in bafilomycin (Fig. 2). As discussed above, we now realize that autophagy creates a rapid flux through the pathway with rapid generation and degradation of autophagosomes. Accordingly, it is possible that the observed level of LC3-II

during lapatanib treatment significantly under-represents the amount of autophagy that is occurring. We propose to repeat this experiment with the addition of bafilomycin over the final 2 h of each incubation to assess the amount of autophagy occurring in a defined time period. It is important to note however, that cells suppressed for ATG12 showed almost no LC3-II but rather the precursor LC3-I was observed (Fig. 8). Accordingly, this experiment shows that we are able to suppress autophagy, at least transiently, with our shRNA constructs.

We have attempted to perform long term growth assays with wildtype cells and those suppressed for autophagy. Incubation with lapatanib very successfully suppressed growth over 10 days Fig. 9. As originally proposed, the culture was static and there was little if any cell death that occurred over this time. However, when we attempted to perform similar experiments in parallel cultures of cells suppressed for ATG5 or ATG12 we were confronted by the issue that the cells no longer repressed these critical autophagy genes. When incubated with bafilomycin, these cells once again showed accumulation of LC3-II (Fig. 10). Hence we were unable to use these cells to test the hypothesis that suppression of autophagy would enhance cell killing.

We also tried an alternate approach whereby autophagy was suppressed with small molecule inhibitors. Neither inhibitor is truly selective. 3-methyladenine has frequently been used to inhibit autophagy because it inhibits the PI3-kinase required for activation of beclin. However, it also inhibits the PI3-kinase that activates AKT, so it may have confounding effects. Indeed, alone 3-methyladenine completely suppresses cell growth (Fig. 9). The second inhibitor is chloroquine which prevents acidification of the lysosomes after fusion with the autophagosome. In this case LC3-II should increase further as its degradation will be inhibited, but the inhibition of lysosomal activity may have many additional effects on the cell. We found that neither inhibitor markedly increased the rate of cell death, which again suggests our original hypothesis maybe disproven. However, we believe that a pure genetic approach is still required to fully test the hypothesis, and we continue to attempt to suppress ATG5 and ATG12 in SKBr3 cells to finally resolve this question.

Task 4: To determine the impact of autophagy in response of cells to trastuzumab *in vivo*
This task required successful completion of Tasks 2 and 3 and as neither of those was accomplished during the time frame of this research, we have not performed any in vivo experiments.

KEY RESEARCH ACCOMPLISHMENTS

- Lapatinib was very effective at inhibiting signaling through AKT and ERK during a 72 h incubation and in inducing autophagy. This was observed in both BT474 and SKBR3 cells.

- Lower concentrations of lapatanib (20 nM as compared to 1 μM) induced only a transient inhibition of ERK signaling, and after 8 h there was dramatic over-stimulation of this pathway. However, this concentration was still effective at inhibiting phospho-AKT and inducing autophagy.

- shRNA targeted to ATG12 was effective at suppressing autophagy over a short period. However, this suppression of autophagy was not retained over adequate time period. This prevented assessment of whether autophagy protected cells from lapatinib-mediated cell death.

REPORTABLE OUTCOMES

An oral and poster presentation of this research project was presented at the 2008 DOD Era of Hope conference in Baltimore. As this was a concept grant with only one year of funding, the experiments have not yet reached the stage where sufficient results have been obtained to submit a full publication. We hope that the ongoing experiments will quickly lead to the anticipated results that will permit publication.

Eastman A. Contribution of Autophagy to the Response of Breast Cancer to Trastuzumab. DOD Era of Hope Conference, Baltimore, 2008 (abstract appended).

CONCLUSION

The results obtained to date are consistent with the original hypothesis that inhibitors of growth factor receptors will induce autophagy. Surprisingly however, we have been unable to confirm that autophagy protects the cells from these drugs which does not support the original hypothesis.

PERSONNEL SUPPORTED

Funds from this grant supported Dr. Alan Eastman (PI; 12% effort, 6 months) and Ms Ryan Montano (Research Technician; 100% effort, 12 months).

SUPPORTING DATA

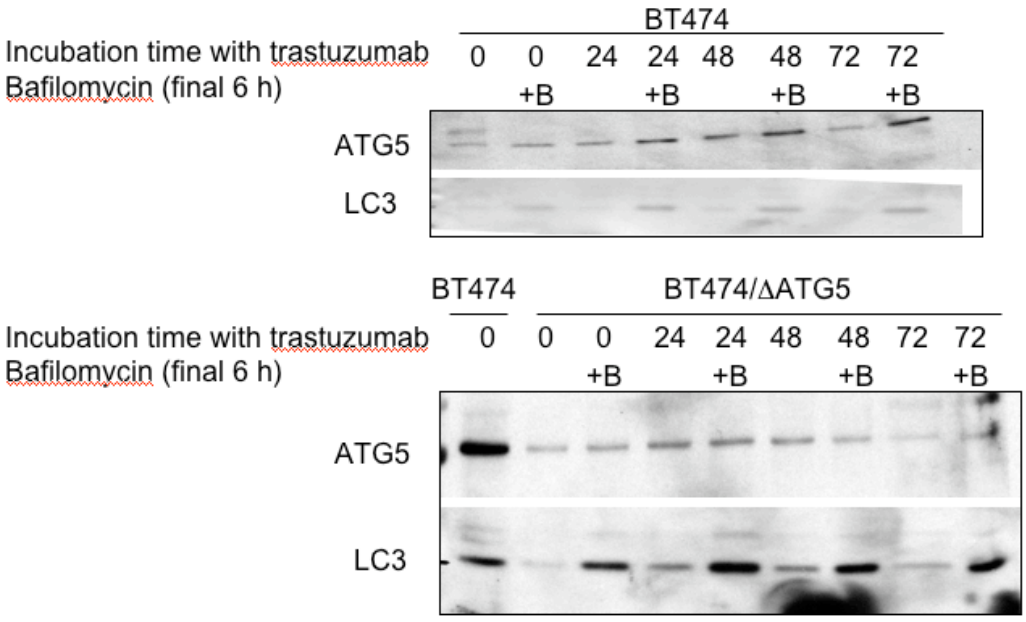

Fig. 1. Impact of trastuzumab and bafilomycin on induction of autophagy in BT474 cells. Top panel: Cells were incubated with 2 μg/ml trastuzumab for 0-72 h with no impact on autophagy as judged by failure of LC3-II to accumulate. A concurrent 6 h incubation with bafilomycin showed the expected accumulation. Bottom panel: shRNA targeting ATG5 markedly reduced ATG5 protein in BT474 cells and suppressed endogenous LC3-II. Trastuzumab had little effect on accumulation of LC3-II, but incubation with bafilomycin showed that the suppression of ATG5 was insufficient to prevent the accumulation.

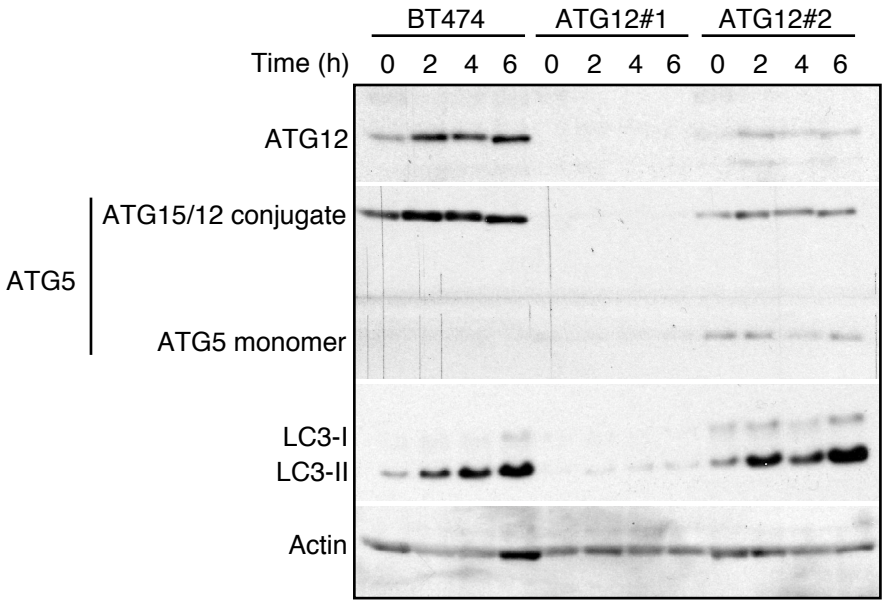

Fig. 2. Induction of autophagy in BT474 with bafilomycin, and suppression of autophagy in cells incubated with shRNA targeting ATG12 (ATG12#1). The ATG12#2 cells were only partially suppressed for ATG12 and show no inhibition of bafilomycin-induced autophagy.

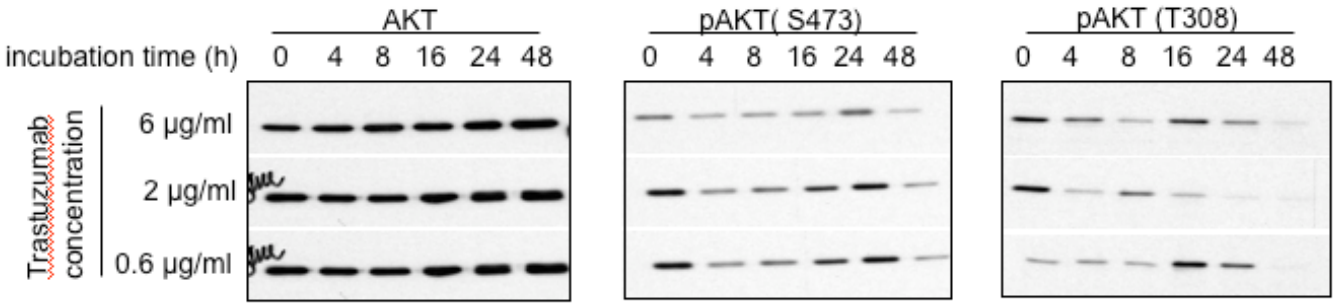

Fig. 3. Failure of trastuzumab to effectively inhibit phosphorylation of AKT. BT474 cells were incubated with the indicated concentration of trastuzumab for 0-48 h and phosphorylation of AKT on serine 473 and threoinine 308 was assessed by western blotting.

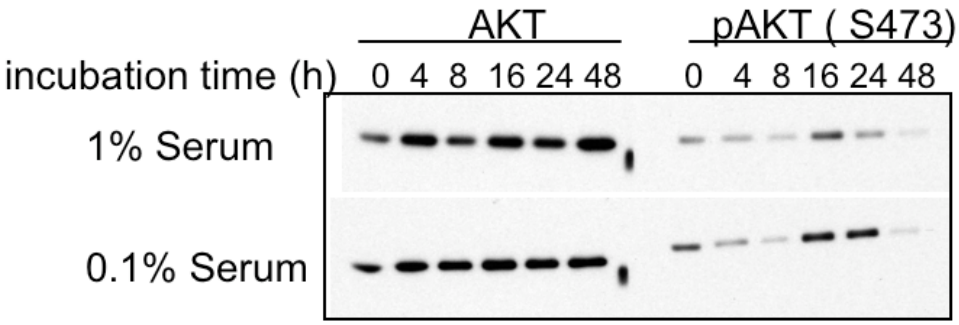

Fig. 4. Lack of impact of serum concentration on trastuzumab inhibition of phospho-AKT. Cells were incubated with 2 µg/ml trastuzumab for the indicated time in either 1% or 0.1% serum. The transient inhibition of phospho-AKT is similar to that observed in 10% serum in Fig. 3.

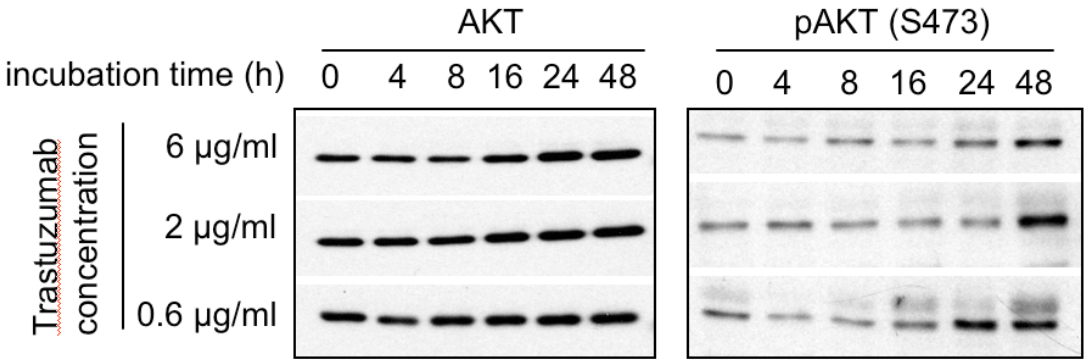

Fig. 5. Failure of trastuzumab to suppress phosphorylation of AKT in ZR75.1 cells. Cells were incubated with the indicated concentration of trastuzumab for 0-48 h and phosphorylation of AKT on serine 473 was assessed by western blotting.

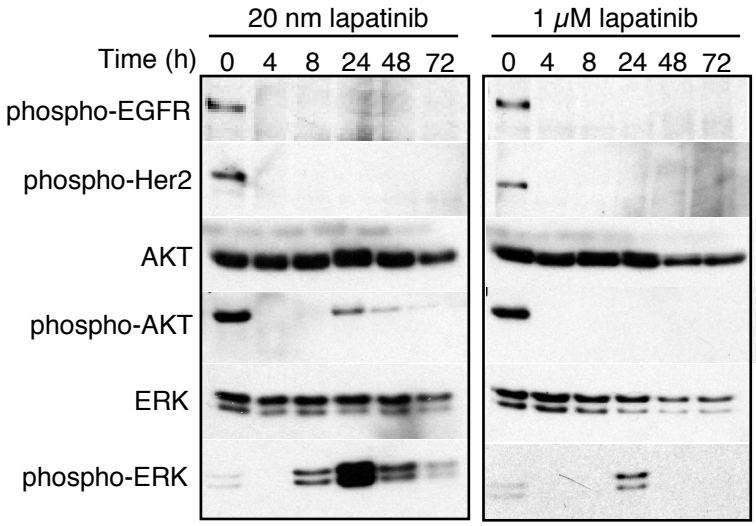

Fig. 6. Inhibition of signal transduction in BT474 cells incubated with either 20 nM or 1 μM lapatinib. Phospho-EGFR and phospho-Her2 were inhibited throughout the 72 h incubation at both concentrations, but cells incubated with 20 nM lapatinib exhibited a dramatic rebound in phospho-ERK by 8 h.

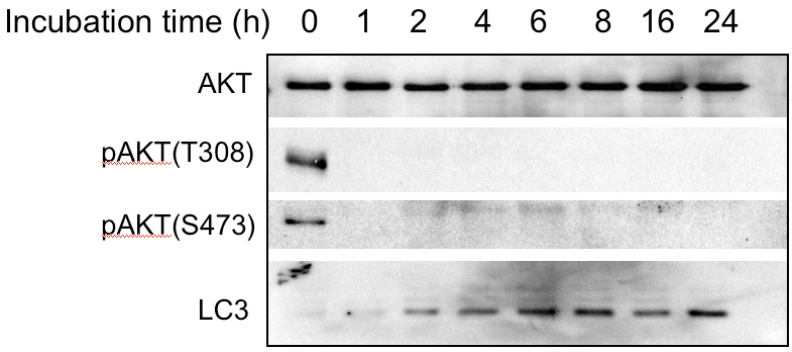

Fig. 7. Lapatinib (20 nM) inhibits phosphorylation of AKT and induces accumulation of LC3-II in BT474 cells

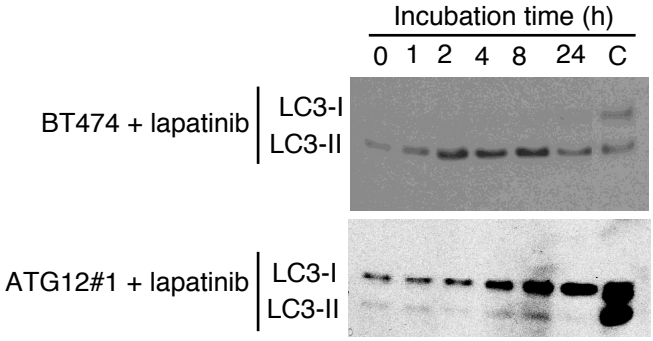

Fig. 8. Induction of autophagy in BT474 cells incubated with 1 μM lapatinib as assessed by accumulation of LC3-II. Cells suppressed for ATG12 showed accumulation of LC3-I but minimal conversion to LC3-II consistent with inhibition of autophagy.

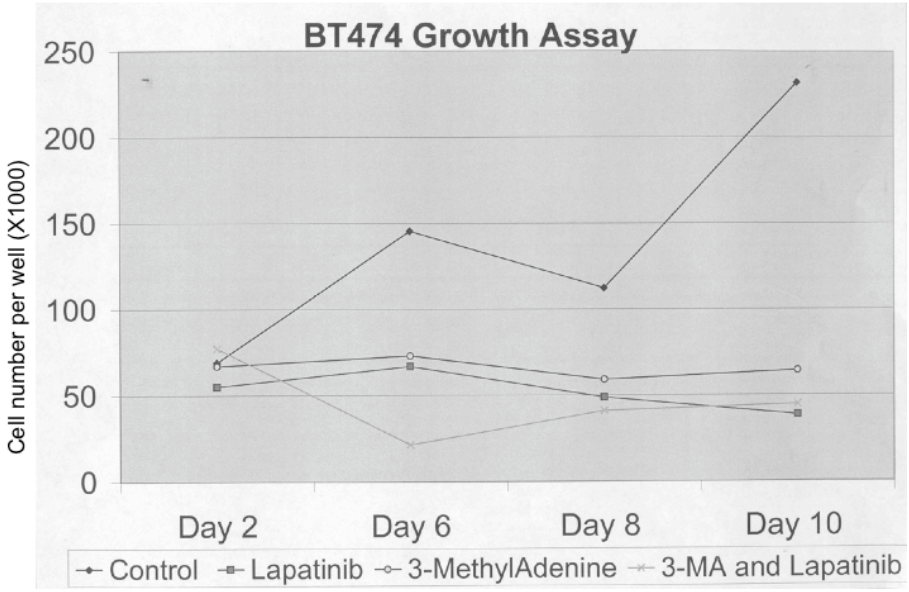

Fig. 9. BT474 cells were incubated in the presence of lapatanib, 3-methyladenine or the combination and growth assessed over 10 days.

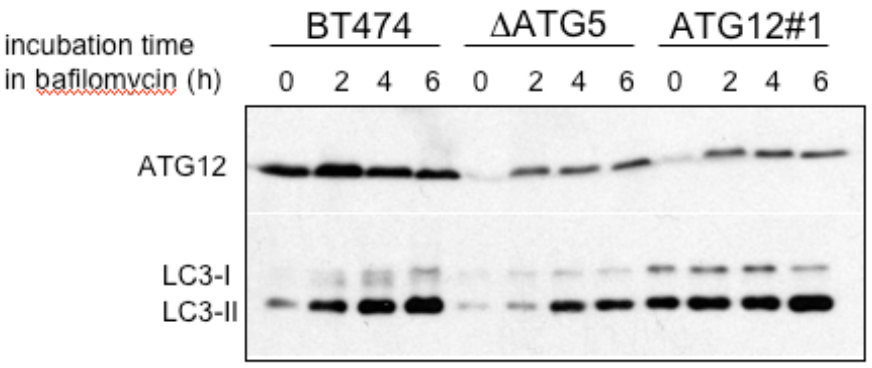

Fig. 10. Recovery of ATG12 in BT474 cells previously repressed for this protein. The same cells shown in Fig. 2 were retested by incubating with bafilomycin. Both ATG12 protein and the lapidated form of LC3 LC3-II accumulated indicating that autophagy had been induced.

Abstract from DOD Era of Hope Conference, Baltimore, 2008.

CONTRIBUTION OF AUTOPHAGY TO THE RESPONSE OF BREAST CANCER TO TRASTUZUMAB
BC062725

Alan Eastman

Trastuzumab (Herceptin®) is an effective therapy for a subset of breast cancer patients whose tumors over-express Her2, although many of these tumors fail to respond, while many others acquire resistance and recur. Continued administration of trastuzumab to resistant tumors can still reduce the rate of tumor growth; hence, patients can continue to benefit for a while. In vitro experiments may reflect this in vivo situation. Specifically, the growth of BT474 breast cancer cells is effectively suppressed by trastuzumab, but it is relatively ineffective at killing the cells. Trastuzumab inhibits the Her2 receptor and thereby decreases intracellular signaling through Akt. Activated Akt is well-recognized as a suppressor of apoptosis, yet in this model, cells survive the inactivation of Akt. Akt, through mTOR, is also a repressor of autophagy, a process by which a cell digests its own cytoplasm and organelles and recycles the constituents when energy supplies are low. Autophagy has been variously considered as an alternate mechanism of cell survival or cell death. Autophagy does eventually result in cell death, but for many days a cell is able to survive with the potential to recover between periods of drug administration. We hypothesized that growth suppression induced by trastuzumab would inhibit Akt and induce autophagy and that this would contribute to survival of tumor cells. Preliminary results have indeed demonstrated that trastuzumab induces autophagy in BT474 cells. Current experiments are directed to a comparison of the efficacy of trastuzumab in cells competent or incompetent to undergo autophagy. We hypothesize that inhibitors of autophagy may be combined with trastuzumab to enhance its therapeutic activity.

This work was supported by the U.S. Army Medical Research and Materiel Command under W81XWH-07-1-0684.